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ALTERATIONS IN SPLENIC AND HEPATIC PROTEIN KINASE C IN SEPSIS AND CHRONIC ENDOTOXEMIA

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INTRODUCTION

Tissue resistance to various hormones, in particular catecholamines, is a fundamental problem in sepsis, that often prevents the successful resuscitation of septic patients (Chernow and Roth, 1986). In order to devise more effective therapy, it is essential to determine the pathophysiology of this tissue resistance. Recently Roth and co-workers have begun to delineate these pathophysiologic mechanisms (Roth and Spitzer, 1987). Their studies have focused on perturbations in systems linked to protein kinase C (PKC). It appears that sepsis and chronic endotoxemia specifically alter signal transduction systems coupled to PKC, resulting in a down regulation of PKC-linked receptors and an attenuation of phosphoinositide turnover (McMillan et. al., 1986; Carcillo et. al., 1988). We postulated that not only would sepsis alter the more proximal portions of these pathways but that sepsis would also modify protein kinase C itself. This study was therefore designed to determine the effects of sepsis and chronic endotoxemia on hepatic and splenic PKC.

METHODS

Animals

Two rat models, both employing Sprague-Dawley rats (200-500g), were studied. In the cecal ligation with

puncture model, animals (N=6) underwent midline laparotomy and the cecum was devascularized, ligated, and punctured with a 22 gauge needle. The control animals (N=6) underwent a sham procedure that included a midline laparotomy without cecal devascularization, ligation, or puncture. The liver and spleen were harvested 18-24 hours post-procedure. In the second model an Alizet 2-ML-1 osmotic pump was implanted subcutaneously (Roth and Spitzer, 1987). Isotonic sterile pyrogen-free saline (N=6) (154 mM NaCl) or Escherichia coli endotoxin (N=6) (026:B6 boiven method, Difco Laboratories, Detroit, MI) were infused into the jugular vein. A non-lethal dose of endotoxin (0.1 mg/100 mg body wt/24 h) was administered. 30 hours after the onset of the endotoxin or saline infusion, the spleen and liver were harvested, frozen on dry ice, and stored at -70^{0}.

Receptor Autoradiography

20 micron thick tissue sections were cut in a cryostat at -20^{0} C, placed on gelatin coated slides, and freeze dried overnight. The slides were incubated in binding buffer and 2 nM ^{3}H-PDBu (phorbol-12,13-dibutyrate) as described by Sando and Young, 1983. 5 micromolar unlabelled PDBu was used to measure non-specific binding. In these studies percent specifically bound counts ranged from 80-90%. After incubation at room temperature for various time periods, the slides were washed in three 30 second changes of ice cold binding buffer containing no ligand. The slides were blown dry and then either scraped off and counted by liquid scintillation or placed against tritium sensitive film (Hyperfilm, Amersham) for in-situ autoradiography. Autoradiographs were developed with D-19 Kodak after a four week exposure. Typically 10 slides from each harvested organ were studied.

RESULTS

In the initial stages of this study the optimal conditions for PDBu binding were defined. First the time course of phorbol ester binding to the tissue sections was examined. (Fig. 1A) PDBu binding reached equilibrium after 120 minutes incubation. For the remainder of the study all slides were incubated for 2 hours. For optimal phorbol ester binding in tissue homogenates, phospholipid such as phosphatidylserine must be added (Sando and Young, 1983). We examined whether the addition of 100 microgram/

ml phosphatidylserine changed PDBu binding (Fig. 1B). There was no significant difference in specifically bound counts when phosphatidylserine was added, implying that adequate phospholipid was present in the tissue sections to obtain maximum binding.

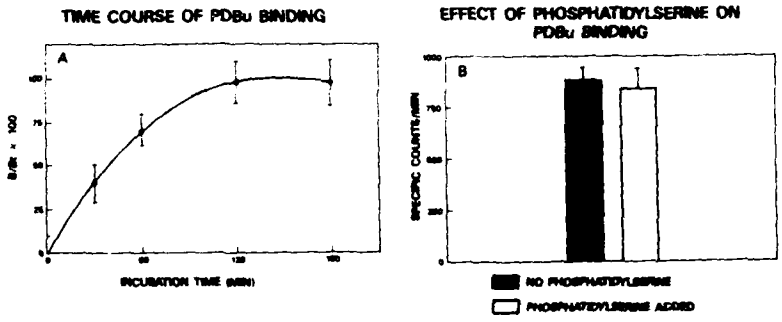

Figure 1. Fig 1A demonstrates the time course of PDBu binding to the tissue sections. B/Bt equals the specifically bound counts at each time point divided by the specifically bound counts at 180 minutes. The means +/- SEM of three triplicate experiments are presented. PDBu binding reached equilibrium after 120 minutes. Figure 1B demonstrates that 100 mg/ml phosphatidylserine had no effect on specific binding of PDBu to the tissue sections. The means +/- SEM of three triplicate experiments are presented.

The in-situ distribution of PDBu binding is shown in figures 2-5. The photographic negatives of the original autoradiographs are presented. Therefore, the lighter the optical density the greater the amount of PDBu bound, and the darker the optical density the lower the amount bound. In each figure there are two representative control sections and two representative cecal-ligation or endotoxin-infusion sections. The control sections in both spleen and liver have a "speckled" pattern of high intensity PDBu binding; in contrast the septic organs have lost this pattern. These sections are representative of the many slides studied. This technique did not allow an exact anatomic or cellular localization of these "speckled" areas.

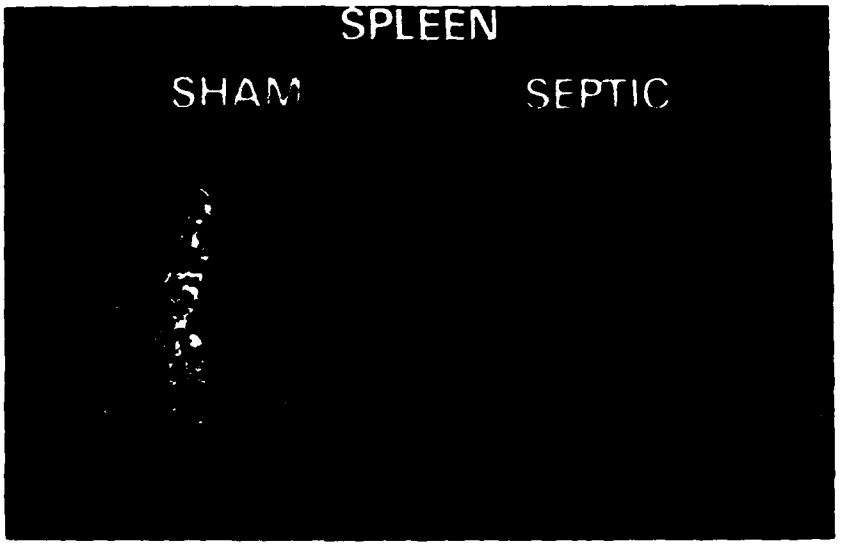

FIGURE 2. The in-situ autoradiographic distribution of splenic PDBu binding in representative sham and septic (cecal ligation and puncture) sections is shown. The control sections have a "speckled" pattern of high intensity phorbol ester binding that is lost in the septic sections.

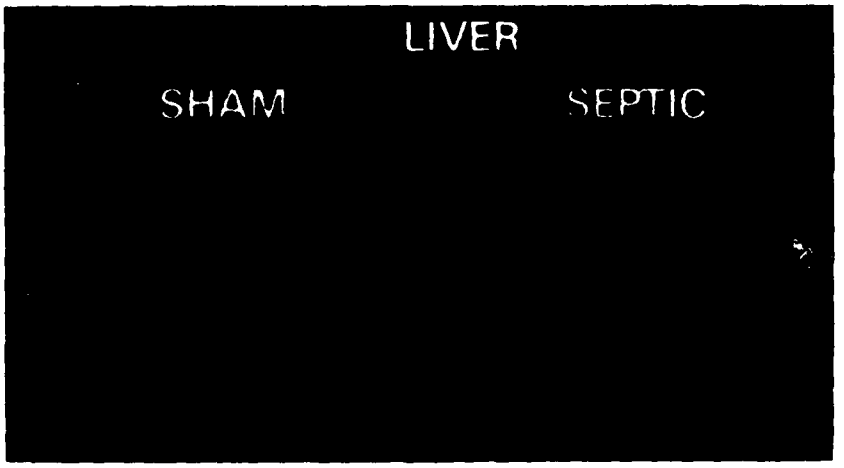

FIGURE 3. The effect of cecal ligation and puncture on PDBu binding within the liver is demonstrated. Again the "speckled" pattern is lost in the control sections.

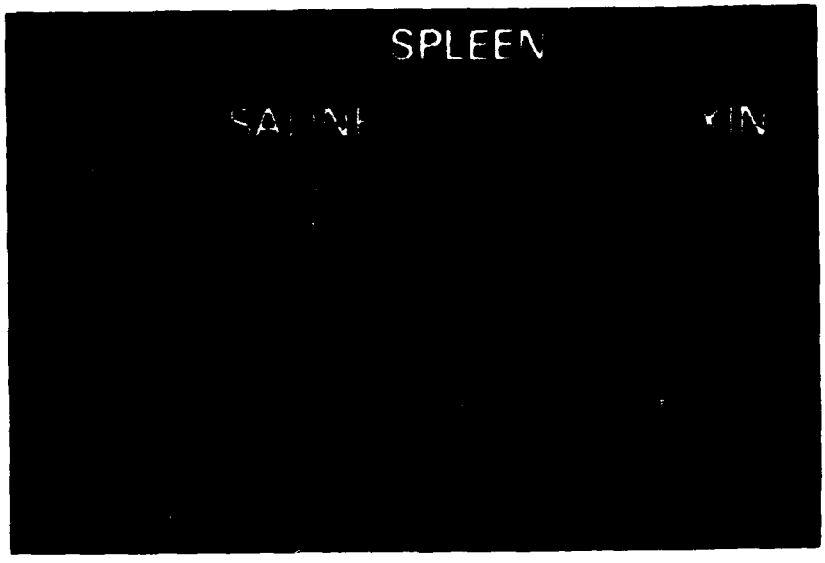

FIGURE 4. The saline-control sections have the high intensity PDBu binding regions that are not observed in the chronic endotoxin sections.

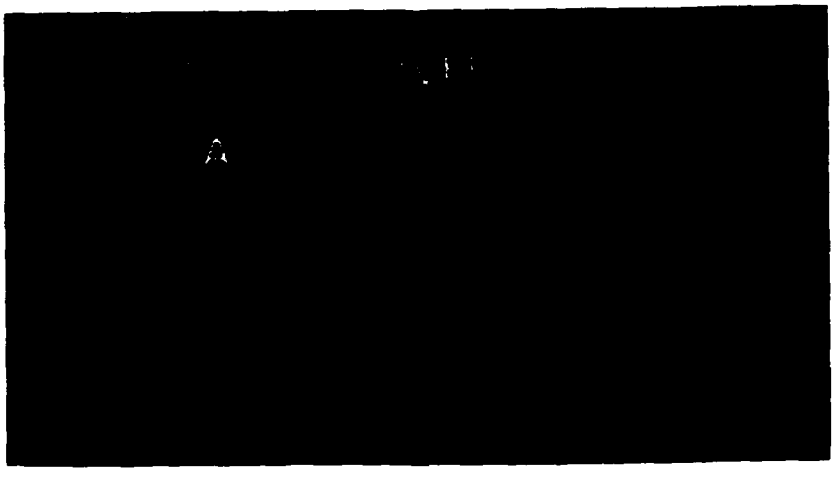

FIGURE 5. The "speckled" areas of high intensity PDBu binding noted in the saline-control sections of the liver are lost in the chronic endotoxin sections.

DISCUSSION

Since the discovery of protein kinase C by Nishizuka's group in 1977, this calcium and phospholipid dependent kinase has been found to play an essential role in the faithful signal transduction of various hormones, growth factors, and neurotransmitors (Takai, 1977; Nishizuka, 1986). Hormones such as norepinephrine, angiotensin II, and vasopressin may mediate their sustained effects by activating PKC (Berridge and Irvine, 1984; Rasmussen, 1986). When these external messengers bind to their target surface receptors they activate phosphoinositide hydrolysis, generating diacylglycerol and inositol phosphates. The inositol phosphates cause the release of calcium from the sarcoplasmic reticulum and the diacylglycerol binds to the putative phorbol ester receptor on PKC. The increase in diacylglycerol and the rise in cytoplasmic calcium activate PKC by translocating it from the cytoplasm to the plasma membrane (Wolf et. al., 1985). Previous studies have shown that the more proximal portions of this pathway are disturbed in sepsis. In a cecal ligation and puncture model, McMillan et. al.,1986, demonstrated that sepsis decreased the number of hepatic alpha$_1$-adrenergic receptors. Similarly, chronic endotoxemia reduces the number of hepatic vasopressin and alpha$_1$-adrenergic receptors without affecting receptor affinity (Roth and Spitzer, 1987). In addition, the synthesis and hydrolysis of phosphinositide bis phosphate is attenuated in sepsis (Carcillo et. al., 1988). Because no studies have examined whether PKC itself is altered in sepsis, the effects of chronic endotoxemia and sepsis on hepatic and splenic PKC were examined.

We elected to assay changes in PKC with an analysis of in-situ receptor autoradiography of hepatic and splenic phorbol ester binding sites. Since phorbol esters specifically bind with high affinity in a 1:1 stoichiometry to PKC, they can be used to detect alterations in protein kinase C (Nichizuka, 1984; Nishizuka, 1986). This technique has been successfully applied to the analysis of phorbol ester receptors as well as many other receptors within the brain (Worley et. al., 1986; Roth et. al., 1987). Using this method, we found a remarkable alteration in the distribution of phorbol ester binding. The "speckled" pattern of high intensity binding noted in the control sections was lost or significantly attenuated in the endotoxin and cecal-ligation

sections. Because only one concentration of PDBu was examined, the change in phorbol ester binding could have been a consequence of either a decrease in affinity for PDBu or an actual decrease in the amount of receptor. A reduction in receptor number could result from a decrement in PKC within the "speckled" areas; however, because the putative phorbol ester binding site can be proteolytically cleaved from the catalytic site, it is possible that decreases in phorbol ester receptor number could be associated with no change or an increase in protein kinase C phosphorylating activity (Parker et. al., 1986).

Although the impact of sepsis on PKC has not been previously studied, several groups have determined the effects of chronic protein kinase C stimulation. Chronically stimulating cells with phorbol esters leads to a diminution in phorbol ester binding, a decline in the quantity of the protein, and a decrease in phorbol responsiveness. Apparantly an augmented rate of PKC degradation causes these changes (Young et. al., 1987). With chronic phorbol ester stimulation, steady state PKC -mRNA levels are constant and the rate of PKC synthesis remains fixed. A similar mechanism may occur in sepsis, since during overwhelming infection, numerous hormones, autocoids and inflammatory factors capable of stimulating PKC-linked pathways are released.

In summary, this study has defined a relatively simple method of delineating phorbol ester receptors within the spleen and liver. Further, it was shown that sepsis and chronic endotoxemia markedly alter the regional distribution of phorbol ester bound within the liver and spleen. The attenuation of PDBu binding within the "speckled" areas could result from either decreased receptor number or reduced receptor affinity. If these alterations in in-situ phorbol ester binding are associated with decreased physiologic PKC activity, then tissue resistance in sepsis may not simply reflect perturbations at the more proximal levels of signal transduction (the receptor and second messengers) but may also result from dysfunction at the level of the protein kinase.

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those of the authors and are not to be construed as reflecting the Navy Department or the Department of Defense. The experiments were conducted according to the principles set forth in the guide for the care and use of Laboratory Animals, DHEW publication (85-23). Supported by Work Unit Number M0095.001.1005.

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DISCUSSION

Dr. Clemens: Your picture of the liver looks kind of intriguing, especially in the normal livers. From here it looked like what you had outlined was the lobular structure of the liver with the light and dark areas corresponding perhaps to the central lobular and periportal areas.

It is well known that these areas receive a very different oxygen tension because of the gradients along the portal circulation, and as a result there are marked differences in even enzyme distributions in these regions. It seems as though what you are getting with either sepsis or endotoxin is a loss of this gradient. Do you think that you could have just provided us proof that the differences that we see in endotoxin and sepsis are because of circulatory changes and have nothing to do with circulating factors?

Dr. Hermiller: The first point about the localization of the PKC or the phorbol binding, it is our impression, as you stated, where the phorbol binding is. We are performing now emulsion studies to help better define that.

As far as whether this is a flow phenomenon, what is certainly an intriguing hypothesis. It centrainly could be.

Dr. Raetz: Have you assayed extracts of the tissues to see if protein kinase C activity has disappeared?

Dr. Hermiller: I have not. Dr. Spitzer may have.

Dr. Judy Spitzer: I am coming with that. We have been looking at PKC activities in the pallet and in the cytosolic fraction of both whole tissue and in digitonin extracted cells. Even though this is a much cruder measure of protein kinase C activity than what Bryan and his co-workers have been doing, still we see a significant alteration in terms of less PKC activity being associated with the pellet--that is the tightly membrane-bound fraction--and more associated with the cytosolic fraction. This is both in terms of absolute activity and in the percentage distribution in liver and in spleen tissue.

Dr. David Millar (NMRI): I just wondered if in these experiments you have looked at cyclic AMP levels, or do you plan to? Because it would be really remarkable if endotoxin would turn out to be one of these agents. It might throw the switch the other way, as it were.

Dr. Hermiller: I have not, and we may talk afterwards, Dave.